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PROGRESS REPORT

(FINAL)

NASA Grant NGR-05-007-205
April 1, 1969 - March 31, 1970

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A great part of the study had to be devoted to further development of the methodology for three-dimensional analysis of the nervous system on the basis of electron micrographs of serial sections. As a result, a fully developed, tested, and standardized methodology now is available for future work.

This work was done in connection with an analysis of a part of the circuitry of the rabbit retina. This analysis has led to interesting results that illustrate the potentiality of this technique.

In addition, some exploratory work was done with respect to the visual cortex of the cat brain. A proper technique for preservation of the visual cortex was worked out and a technique to localize microelectrode tips in the tissue in connection with electron microscopy was partially worked out.

METHODOLOGY

To illustrate the elaborate combination of technical problems associated with this kind of analysis, the following review of the requirements that must be fulfilled for such studies will be presented. This will give some basis for realizing the importance of the systematic work on the development of the methodology which has been a part of this project.

For the rigorous and exacting type of circuitry analysis aimed at in this study, the following requirements must be fulfilled.

1. It must be possible to analyze a sufficiently large volume of tissue to make it possible to identify basic properties of a circuit. This means that it must be possible to cut rather long series of serial sections.

2. It must be possible to analyze very complex networks of nerve processes.

3. It must be possible to pursue the three-dimensional analysis with the input of a reasonable amount of time.

These three requirements can all be fulfilled. Requirement 1 was already satisfied in the 1950's when in my laboratory in Stockholm the serial sectioning technique was developed to allow mass sectioning of serial sections. As examples of what has been achieved by my collaborators and former students, I would like to refer to the work of E. Andersson-Cedergren and U. Karlsson, which involved cutting serial sections through a depth of 0.7 mm with an average thickness of the sections of 700 A. These researchers collected about 10,000 serial sections through stretch receptors in the frog toe muscle. U. Karlsson reconstructed in three dimensions the entire soma of two neurons in the lateral geniculate body of the rat from series consisting of more than 500 sections each. L. Barajas has reconstructed the juxtaglomerular apparatus in the rat kidney in several glomeruli in series of more than

500 sections. In my own work, the cutting of series of 500 - 700 sections in single runs has been more or less routine. Longer series of sections are obtained by repeated serial sectioning.

With the exception of the photoreceptor cell terminals in the outer plexiform layer of the retina, the useful section thickness is 1,000 A. Since the dimensions of the neural processes are considerably longer than 1,000 A, a process will, irrespective of its orientation with respect to the plane of the section, be represented by profiles in several sections. This means that a loss of one or even two consecutive sections in a series does not ruin the chances to trace the processes. This is very important to realize because long series of sections are difficult to obtain without losses of individual sections.

Such losses are mainly encountered in connection with the breaking up of a ribbon of sections into sequences that are of a length corresponding to the length of the slit in the specimen slit aperture used to introduce the sections into the electron microscope specimen holder. The maximal length of this slit is in turn dependent on the range of movement of the microscope stage.

In order to reduce the risk for losses of this kind, a special object stage was ordered from the AEI company in connection with the purchase of an electron microscope of their manufacture. The basic design of this electron microscope allowed the introduction of an object stage for long-range movement. After many trials, the AEI company came up with a perfect solution to the problem. It is now possible to analyze ribbons of sections that are 25 mm long. This meant that the ribbons do not have to be broken up by any manipulations since they usually spontaneously break into pieces shorter than 25 mm. The losses of sections can therefore be reduced drastically, and the serial sectioning can be speeded up.

With respect to the length of the series of sections that is required to make the serial sectioning useful, the following points will be stressed:

1. In a nervous center, we consider that circuits are present which are designed for a particular type of data processing which requires (a) a particular selection of types of neurons, (b) a particular number of neurons of each type, and (c) a particular pattern of connections between these neurons. Such a circuit will be referred to as an elementary circuit. In a nervous center, different types of data processing are likely to occur in parallel. Therefore, we can have several different types of elementary circuits.

2. The capacity of a nervous center to process incoming data we can assume to be determined by the number of elementary circuits present in the center. It is therefore reasonable to assume that the elementary circuits are repeated many times. The gross structure of the brain as revealed by means of classical neuroanatomy clearly indicates such a repetitious arrangement of patterns of neurons.

On this basis, it is possible to analyze the neural circuitry in steps, provided that the analyzed regions overlap sufficiently to allow recognizing particular circuitry patterns

in the overlapping regions. Such a step-wise analysis makes it possible to analyze even large nerve centers on the basis of series of sections that cover only part of the volume of the center. It is, however, practical to try to cover as much as possible of the circuitry of a center in each series, and the ideal must be to cover the whole center by one continuous series of sections. This is, however, not a requirement.

To improve the chances to obtain very long and perfect series of sections, a new ultramicrotome has been designed based on a radically different principle as compared to existing microtomes. This microtome is now close to being ready for testing. Only the drive mechanism remains to be built.

In regard to the second requirement mentioned above, the situation became very critical in 1966 because the technique used at that time was not satisfactory to allow analyzing the circuitry of the β-type photoreceptor cells of the rabbit retina in a complete way. In fact, the most difficult situation for a three-dimensional analysis was faced in this case. The reason was that the processes contacting the β-type photoreceptor cell terminal were very thin, the thinnest measuring only 600 A in diameter, and these processes were closely packed without any interposed Müller's cell cytoplasm. They furthermore were intertwined in a most complex fashion.

The dimensions of these processes are about ten times smaller than the dimensions of nerve processes in other parts of the nervous system. Instead of using 1,000 A thick sections, it was necessary to analyze 500 A thick sections since a process must be cut through to avoid its being lost for tracing. But since a 600 A thick process will appear cut through in only one single 500 A thick section, no section in the series can be allowed to be missing within the critical region.

The situation was such that it exposed the method for three-dimensional analysis to a most rigorous test. If it would not be possible to solve the technical problems, three-dimensional analysis would only be useful for the analysis of circuitry that was not too complicated.

The methodology to trace processes was improved and eventually a complete reconstruction of all connections to a β-type photoreceptor cell terminal was carried through successfully. Due to the complexity of the structure, this reconstruction was made to represent in a precise way the shapes and dimensions of all processes. An idea of the time required for such a reconstruction is offered by mentioning that after the final methodology had been worked out, it took three years of practically full-time work to make the reconstruction.

This work clearly showed that it is highly unlikely that any circuitry in the nervous system is too complicated to be analyzed by means of this technique. Other parts of the retina and centers in the brain are easy to analyze in comparison to this particular part of the retina.

This experience with model building emphasizes the importance of requirement 3. Considerable work has been done to cut down the time required for three-dimensional analysis. As mentioned above, the new type of object stage for our electron microscope has allowed us to save time spent on serial sectioning.

One of the most time-consuming operations involves handling of photographic material in connection with taking electron micrographs of the serial sections. In fact, more than 90% of the time spent on electron microscopy of this kind is consumed by handling photographic material when using a conventional electron microscope. To reduce this time, a camera was built for our AEI electron microscope which can be loaded with a 500-foot, 70 mm roll film. This allows taking 1,500 pictures without reloading and reduces the time spent on electron microscopy to one-tenth of that spent when using a conventional electron microscope.

The next time-consuming operation involves tracing the nerve processes in three dimensions. The precision in this operation was greatly improved by making prints of the electron micrographs on transparent film which then could be superimposed for tracing. It now seems possible to use the original electron micrographs without printing, but this requires the development of particular equipment.

Storing the large amount of information obtained from the tracing represents another major time-consuming step in the procedure. The simplest and fastest way to store the information was worked out and was found to be the building of a physical model in which each nerve processes is represented by a thin wire. The wire is stiffened by pushing small pieces of plastic tubing onto the wire. The length of these pieces corresponds to the thickness of the section. This way, a mechanically stable three-dimensional model with a linear representation of the nerve processes is built in which contacts between the processes are marked.

The position of a process in space is at each level during the reconstruction determined by its position relative to a coordinate system. A square coordinate system is drawn on top of the large viewing table which makes it possible to assign particular coordinates to any profile of a nerve process in the electron micrographs arranged in a montage on the tabletop. Since the model is built on the basis of a corresponding coordinate system, the proper position of the process in the model can be found and the process can be accordingly extended as the reconstruction proceeds.

This method is considerably faster than the use of a computer to store the information. In the latter case, both feeding the information into the computer and retrieving the information are more time-consuming than building the model and looking at the model in order to analyze the circuitry.

With this work on the development of the methodology for three-dimensional analysis of the nervous system, we are now in a situation where a standardized and tested methodology is available which allows such work to be done with a reasonable investment of time even if such studies still will be time-consuming. Extensive three-dimensional analysis of nervous

centers can therefore be evaluated as feasible to perform. This means, in fact, that a new era in the analysis of the nervous system has started.

THE PILOT PROJECT

The methodology for three-dimensional analysis of the nervous system based on electron micrographs of serial sections was worked out in connection with a study of a part of the circuitry of the outer plexiform layer of the rabbit retina.

The aim of this study was to search for the circuitry responsible for directional selectivity of the response of certain ganglion cells in this retina as discovered by Barlow and Hill (1963). This circuitry appeared particularly suitable for such an analysis since the type of input into the circuit was known as well as the output. The directional selectivity means that the ganglion cell involved discharges vigorously when an image moves over its receptive field in one direction (the "preferred direction") whereas a movement of the image in an opposite direction ("null direction") evokes little or no response. One-third of the ganglion cells in the rabbit retina are directionally selective units.

From an anatomical point of view, the rabbit retina appears fairly simple with a tremendous convergence of the circuitry toward a rather limited number of ganglion cells. The rabbit furthermore is colorblind, and anatomically the retina is a pure rod retina. This implies that the circuitry of the rabbit retina might be fairly simple in spite of the fact that the data processing is advanced.

Directionally selective neurons have been observed in retinas of animals with a panoramic type of vision, while in animals with stereoscopic vision such units have been found in the brain. This does not, of course, mean that the directional selectivity is not coded for already in the retina in the latter case. It only means that units specialized for this function are located at different levels of the nervous system.

A directional selectivity in ganglion cells is activated even when the movement of the image over its receptive field is confined to a distance of about 50 μ in the preferred-to-null direction. This means that the input circuitry for directional selectivity must be duplicated many times over the receptive field (Barlow and Hill, 1963). This means that this circuitry has dimensions that are suitable for three-dimensional analysis even when dealing with rather limited volumes of retinal tissue and that a similar circuitry pattern must be repeated with a rather high frequency in the retina.

The three-dimensional analysis has revealed a circuitry pattern which might well represent the basis for directional selectivity.

The analysis used the terminal of one β-type photoreceptor cell as a starting point. All processes contacting this terminal were traced through all sections in the series. Due to the special requirements with respect to section thickness and absolute completeness of the

series in this particular case, the volume of tissue that was analyzed was limited. The series consisted of 89 perfect sections chosen from a series consisting of more than 700 sections. The area covered by the sections was confined to 0.2 x 0.03 mm.

The circuitry pattern that can be correlated to directional selectivity is represented by one bipolar cell process which contacted three photoreceptor cell terminals, two of the β-type and one of the α-type. All these three terminals were lined up in a west-to-east direction along a straight line. This bipolar cell dendrite furthermore sent off two processes that contacted three horizontal cell processes, all of which approached the dendrite from north, that is, perpendicular to the direction along which the three contacted terminals were distributed. In addition, a fourth horizontal cell process approaching from north sent off a process contacting this bipolar cell dendrite.

Of these four horizontal cell processes showing particular contact relations with the bipolar cell dendrite, three ended after contacting the dendrite without contacting any photoreceptor cell terminal located south of the dendrite. This is of importance since it is likely that the horizontal cell processes conduct in both directions depending upon the state of illumination of different areas of the retina. The ending of the horizontal cell processes here would mean that the bipolar cell dendrite could only be influenced by these horizontal cells in one direction, from north.

Of all ten bipolar cell processes contacting the β-type photoreceptor cell terminal, this was the only bipolar cell process that sent off any branches to contact adjacent horizontal cell or bipolar cell processes. It behaved, therefore, in an unique way. The contacts with the horizontal cells were furthermore a systematic contact with all horizontal cell processes approaching the bipolar cell dendrite from one particular direction. This direction was furthermore perpendicular to that along which the contacted photoreceptor cell terminals extended. This certainly shows a systematic pattern of contact relations.

That this pattern could be the basis for directional selectivity becomes obvious if we assign an inhibitory function to the horizontal cells. This would mean that inhibition of the bipolar cell would be confined to illumination of an area north of the bipolar cell dendrite while no comparable inhibition would be associated with the illumination of an area south of the dendrite. The image of a light spot moving from north over this part of the retina would then evoke an inhibition of the bipolar cell through the horizontal cell processes and an inhibitory wave would advance toward the bipolar cell dendrite as the image of the light spot moves toward the photoreceptor cells contacted by this bipolar cell. The effect of the light-stimulated photoreceptor cells on the bipolar cell dendrite would therefore be suppressed by this inhibition.

If the image of the light spot moves from south to north, on the other hand, the light would hit these photoreceptor cells without the contacting bipolar cell dendrite being exposed to the same inhibitory influence. This would therefore be the preferred direction while the north-to-south direction would be the null direction. The lining up of the photoreceptor cells contacted by the bipolar cell in a west-to-east direction would mean that maximal

effect on the bipolar cell dendrite would be obtained when an edge between dark and bright areas in an image were oriented parallel to this direction, thus affecting all receptor cells simultaneously. This arrangement could contribute to discriminating against movements of the image in east to west and in west to east directions.

The analysis furthermore revealed a basic circuitry pattern with respect to bipolar cells and horizontal cells connecting the β-type photoreceptor terminal. A characteristic feature of photoreceptor cell synaptic connections is the invaginated endings associated with a synaptic ribbon located in the cytoplasm of the receptor cell terminal. These invaginated endings form a precise pattern according to which a pair of horizontal cell endings is located in the deepest part of the invagination and is rather large with comparably large surface areas contacting the receptor cell terminal. These two endings are in this deep part of the invagination completely separated by a septum formed by a fold in the plasma membrane of the terminal. The synaptic ribbon is located in this fold. Further distally in the invagination, the two horizontal cell endings are in mutual contact. This is in a narrow "neck" of the invagination. Here mostly one, but in some areas two, thin endings of bipolar cell dendrites contact the two horizontal cell endings. These dendrite endings furthermore make extensive contact with the receptor cell surface.

The analysis revealed that the two horizontal cell endings of the pair originate from different horizontal cells and that these horizontal cell endings extend in mutually perpendicular directions.

This synaptic ribbon complex must have a very precise functional significance because it is a constant feature of photoreceptor cells and it has a very precise morphology.

The following functional interpretation appears reasonable. One important function of the retinal circuitry is to adjust the state of accommodation through lateral inhibition, the neural part of the accommodation mechanism. This accommodation is adjusted to reflect the average illumination of the retina and to be less affected by local variations in the illumination of the retina. This requires that the lateral inhibition is determined by the level of illumination of large areas of the retina. The first step in adjusting the structural organization of the retina to this requirement is represented by the large size of the horizontal cells and the direct electric coupling of these cells. The second step involves numerous small processes extending between horizontal cell processes of an intermediate size which could contribute to elimination of differences in the degree of polarization of these processes, thus washing out differences due to variations in illumination with smaller areas of the retina.

A third step would be to secure that the inhibitory influence of the horizontal cells on a particular bipolar cell would be as representative as possible for the overall level of illumination of the retina. The way this appears to be done is by connecting each bipolar cell ending to two horizontal cell endings representing the level of illumination in two perpendicular directions out from the receptor cell.

The connection between the horizontal cell endings and the bipolar cell endings in the synaptic ribbon complex is therefore interpreted to allow inhibiting influences on the

bipolar cell dendrite by the horizontal cells. The contact between the two paired horizontal cell endings in the synaptic ribbon complex allows these two endings to affect the bipolar cell ending at areas located as closely arranged as possible and also could make it possible for mutual interaction between the two horizontal cell endings. This way, the influence on the bipolar cell ending would correspond to that represented by an average of the influence the two horizontal cell endings could exert.

The large size of the endings of the horizontal cells in connection with the synaptic ribbon complex makes it justifiable to interpret these endings to be postsynaptic to the receptor cell.

The synaptic ribbon is arranged like a shield insulating the two end chambers of the invagination. It is proposed that it might play a role as a structure that is of importance for the current flow through the receptor cell at the synaptic ribbon complexes.

The complexity of one single β-type receptor cell synaptology is illustrated by the following description. This terminal was associated with 16 synaptic ribbon complexes. Twenty-four horizontal cell end branches extended to these synaptic ribbon complexes, and eight of these end branches were associated with two adjacent synaptic ribbon complexes. All these end branches originated from 8 different horizontal cells. Nineteen end branches from 6 bipolar cell dendrites contributed endings associated with the synaptic ribbon complexes.

In addition to synaptic contacts in connection with the synaptic ribbon complexes, there were other types of contacts. One bipolar cell process contacted the terminal through two deeply invaginated endings. In addition, this process contacted some of the horizontal cell endings associated with the synaptic ribbon complexes but laterally to the complexes and frequently at lateral extensions of the horizontal cell endings. The endings of this bipolar cell process were full of synaptic vesicles as densely arranged as the synaptic vesicles in the cytoplasm of the receptor cell terminal.

This process was traced to four other β-type photoreceptor cell terminals, and it made the same type of contact with all of them.

If we call the synaptic ribbon complex contacts as contacts of type I, the contact relations of the bipolar cell process can be called a type II contact.

The basal surface of the β-type photoreceptor cell terminal has a concave shape. In this deep concavity and also further in a vitread direction, all processes contacting the terminal are found closely packed while branching. The form a whorl of curved processes, and the branches are intertwined in a complex fashion. This region will be referred to as the "subsynaptic neuropil."

In the subsynaptic neuropil, a third type of contact relation was observed. One bipolar cell process was located in the center of this neuropil and extended short processes

which in a systematic way contacted all branches of practically all other bipolar cells and the majority of the horizontal cell branches. This process ended with two deeply invaginated endings, one of which made a lateral contact with a synaptic ribbon complex. The cytoplasm of this process contained large numbers of dense granules and some synaptic vesicles.

This process was traced to two a-type photoreceptor cells where it sent off end branches contacting the processes entering the invagination before their entrance into it, and the surface of the receptor cells lateral to the invagination.

It appears justifiable to classify the contact relations of this process as being different from those of the other processes because of its special relationships to the components of the subsynaptic neuropil. We refer to this type of contact as type III.

Other bipolar cell processes contacted the terminal with endings received in shallow invaginations not associated with the synaptic ribbon complexes and without any particular relationships to any other processes. These contacts can be classified as type IV contacts.

One horizontal cell process contacted mainly the processes in the subsynaptic neuropil and sent off a tiny end branch contacting the surface of the terminal without being received in any invagination. This would be the type V contact.

By tracing the processes to other photoreceptor cell terminals, it was possible to establish that with few exceptions a process made the same type of connection with all these terminals.

Altogether, this β-type photoreceptor cell terminal was contacted by 19 different neurons, nine horizontal cells, and 10 bipolar cells.

One conclusion from the analysis appears justifiable to draw. The contacts between the β-type photoreceptor cell and bipolar cells do not appear to be a simple transfer of information only modulated by the presence of horizontal cell endings to adapt to different degrees of illumination. The β-type photoreceptor cell terminal with its subsynaptic neuropil appears more like a rather complex piece of circuitry where the possibility cannot be ruled out that bipolar cells could function as presynaptic elements. Particularly the type II and type III connections appear reasonable to suspect as playing such a role.

If we add to this the lateral connections extending from the β-type photoreceptor cell terminals and contacting all surrounding terminals, the β-type cell stands out as a photoreceptor cell with very specialized and involved function in the circuitry of the retina.

The visual cortex of the cat brain

The analysis and the development of methodology reported above has been so time-consuming and of such a basic importance for the continuation of this type of analysis that only limited time has been spent on the visual cortex.

The method to preserve the brain tissue has been worked out. Perfusion through the carotid artery or through the heart with a glutaraldehyde solution (short-time), followed by an osmium tetroxide solution, was found to be appropriate.

For the localization of the microelectrode tips, the first problem is to limit the volume of tissue within which the tip should be searched for. This was done by fixing the brain with the electrodes in situ and serial sectioning the visual cortex for light microscopy. On these sections, the tracks made by the electrode could be observed, and the sections closest to the tip could be selected. These sections were then reembedded in a plastic and sectioned for electron microscopy.

The next step is to find the most useful label for electron microscopy to localize the tip. This work has not been pursued further due to lack of time.

REPORT OF INVENTIONS

The new design of ultramicrotome described briefly on page 4 of this report may possibly be patentable. A report of this device has been submitted to the University of California Office of Patents, and an investigation of the patentability is being conducted by that office. If the device is patentable, a complete report will be forwarded to NASA.